# CHIMERIC PEPTIDE IMMUNOGENS

This application claims priority from the provisional application No. 60/202,328, filed May 5, 2000 in the United States Patent and Trademark Offic.

## FIELD OF THE INVENTION:

The invention is related to chimeric peptides having immunogenic efficacy, comprising a hormone epitope and promiscuous helper T-cell epitope for the production of high titers of anti-hormone antibodies.

## BACKGROUND OF THE INVENTION

The success of an antigenic composition is linked to its immunogenicity, that is, the ability to produce a sufficiently high titer of antibodies to react or bind with the target antigen or so as to neutralize its effects. The immunogenicity depends on the effectiveness by which the antigen causes the body's immune system to mount a response which can be generally assessed on the basis of the antibody titer in the blood of the immunized animal or mammal including the human.

Antigenic formulations can be prepared for antigens of low immunogenicity with constructs or mixtures of an immunomimic epitope of the target antigen and an immunogen not related to the target antigen so as to generate a strong immune response against the entire immunogenic construct or mixture so as to be effective against the specific target antigen.

In order to enhance or potentiate the immune defense system, so-called adjuvants in the form of oily substances and other potentiating and emulsifying agents are added to the antigenic formulations. In general, the adjuvant is mixed into the immunogenic emulsion formulation and simultaneously delivered with the antigen in the same administration, e.g., by injection. Specifically, antigenic formulations have been enhanced to target less immunogenic microorganisms or viral pathogens by the addition of so-called adjuvants comprising immune response-stimulating killed microbial cells, particles or fragments thereof. Moreover, immunogenic compositions may contain carrier components, including emulsions, liposomes, microparticles and implantable vehicles which may be metabolizable.

Immunization technology has been applied as a biological modifying means to immunize against various soluble and insoluble animal or human self-antigens, which are not normally recognized by the individual host's own immune defense, but which may be

rendered immunogenic so as to stimulate or potentiate the individual's own immune response system. The self-antigens may include the surfaces of certain cells which are malfunctioning or malignant, and small proteins, enzymes or intercellular signals, such as, e.g., hormones or other factors, and/or their cognate receptors, whether normal or deficient. The lack of immunogenicity of these self-antigens has been often overcome by complexing or linking the non-immunogenic self-antigens with a pharmaceutically acceptable, i.e. non-toxic, immunogenic carrier so as to produce antibodies capable of binding, thereby neutralizing, the self-antigen of the subject animal or human patient.

The immunological methods can be used for example in the therapeutical hormone control or regulation and the treatment of patients afflicted with a disorder or disease.

Some immunogens suitable for hormone-regulation comprise hormone immunomimicking molecular moieties which are conjugated or fused to immunogenic carriers, such as, *e.g.*, proteins, or peptides or complex polysugars. The immunogenic constructs are usually administered as either an oil-in-water or a water-in-oil emulsion, containing an adjuvant capable of stimulating or potentiating an immune response.

An immune response is typically measured in terms of the production of specific anti-hormone antibodies. The hormones and cognate receptors which are targeted for control by the immunological methods are directly neutralized or inhibited by the antigen-binding reaction of circulating hormone specific antibodies elicited by the injected immunogenic constructs.

For example, an anti-hormone immunogen has been constructed to affect the regulation of the gonadotropin releasing hormone (see co-assigned U.S. Patent 5,688,506). The Gonadotropin Releasing Hormone (abbreviated "GnRH", also known as Luteinizing Hormone Releasing Hormone, abbreviated "LHRH"), is of central importance to the regulation of fertility. Johnson M et al., *Essential Reproduction*, 3rd Edn. Blackwell Scientific Publications (1988). In both males and females, GnRH is released from the hypothalamus into the bloodstream and is transported through the bloodstream to the pituitary, where it induces the release of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), by the gonadotrophs. These gonadotropins, in turn, act upon the gonads, inducing steroidogenesis and gametogenesis. Steroids released from the gonads into the circulation subsequently act upon various tissues. This gonadotropin related hormonal cascade can be halted by the neutralization of the biological activity of GnRH. Fraser H.M., Physiological Effects of Antibody to

Lutenizing Hormone Releasing Hormone, *Physiological Effects of Immunity Against Reproductive Hormones*, Edwards and Johnson, Eds. Cambridge University Press (1976). As a consequence of GnRH neutralization, the gonadotropins and gonadal steroids are not released into the blood, and their biological activities are curtailed or eliminated by the direct and indirect action of specific anti-GnRH antibodies. By eliminating the physiological activity of GnRH, the cascade of hormonal regulation of fertility is interrupted and gametogenesis ceases. Consequently, GnRH neutralization halts the production of gametes. Thus, GnRH neutralization is an effective means of contraception.

A number of important diseases are affected by gonadotropins and particularly gonadal steroid hormones. Such diseases include breast cancer, uterine and other gynecological cancers, endometriosis, uterine fibroids, benign prostatic hypertrophy and prostate cancer, among others. Removal of the gonadal steroid hormonal stimuli for these diseases constitutes an important means of therapy. An effective method of accomplishing this is by immunologically neutralizing GnRH, to thereby eliminate or inhibit production of GnRH dependent gonadal steroids that induce and stimulate these diseases. McLachlan R.I. et al. Clinical Aspects of LHRH Analogues in Gynaecology: a Review, *British Journal of Obstetrics and Gynaecology*, 93:431-454 (1986); Conn P.M. et al. Gonadotropin-Releasing Hormone and Its Analogs, *New England Journal of Medicine*. 324:93-103 (1991) and Filicori M. GnRH Agonists and Antagonists, Current Clinical Status. *Drugs*. 35:63-82 (1988).

Since GnRH has the same amino acid sequence in all mammals (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly$NH_2$, SEQ ID NO: 1 in the Sequence Listing), it is presumed that a single immunogen would be effective in all mammalian species, including humans. An anti-GnRH immunogenic construct, comprising the GnRH immunomimic domain in the form of peptide analogues, may be linked or conjugated to a carrier protein which is effectively immunogenic, such as, *e.g.*, diphtheria toxoid, tetanus toxoid, keyhole limpet hemocyanin, bovine serum albumin, Hemophilae pertussis extracts or filamentous Amycolata extracts. Consequently, the immune response to the GnRH-vaccine will be mostly directed against the carrier protein and secondarily, the attached hormone epitope moiety. In general, as an alternative approach, the immunogenicity of the immunomimic peptide can be enhanced by chemical modification with diazosulfuric acid groups.

Various anti-GnRH immunogenic compositions have been useful for producing

specific anti-GnRH antibodies. Immunogenic conjugates of GnRH-immunomimic epitope peptide and immunogenic protein carriers have been used for immunization of vertebrate subjects against the hormone, GnRH (U.S. Patent No. 5,688,506).

As another example, anti-hormone immunogens have been constructed to affect or inhibit the activity of the stomach hormone gastrin, in particular, the major forms of gastrin, gastrin G17 and gastrin G34 (see U.S. Patents 5,023,077, and 5,468,494). It has been found that especially G17 is involved in gastrointestinal disorders and diseases such as gastroesophageal reflux disease, gastric and duodenal ulceration and cancer.

However, it has been found that perhaps due to the comparatively huge size of the attached immunogenic carrier proteins, the immunization of the conjugate can induce anti-epitope specific suppression of the antibody (Sad et al. Immunology, 1985, 74:559; Schutze et al. J. Immunol, 1985, 135:231). Therefore, much smaller immunogenic proteins have been tried. Accordingly, short synthetic T-helper epitopes have been introduced to replace the large carrier molecules in conjugates to improve the efficacy of the anti-hormone or self antigenic immunogen. Sad et al. (Vaccine 1993, 11:1145-1149) synthesized peptides from DT and universal or highly promiscuous T-helper epitopes from TT (829-844 amino acids, SEQ ID NO: 2) or CSP (378-398 aa; SEQ ID NO: 3) in order to try to minimize genetic restriction of the immune response. To be effective, the GnRH vaccines of Sad et al. required Freund's Complete Adjuvant.

Gosh et al. (Int. Immunology, 1999, 11:1103-1110) reported that some synthetic LHRH (GnRH) chimeric vaccines elicited an immune response for sterilization of mice. However, the promiscuous helper T-cell (Th)-epitope candidate T1 (TT sequence 947-967 aa, SEQ ID NO: 4) was not regarded promiscuous enough to be applicable for a large number of animal species. It was also reported that in a shift, antisera from second bleedings reacted significantly with the anti-Th epitope (T2) and much less with the LHRH antigen.

## SUMMARY OF THE INVENTION

The present invention provides to immunogens comprising a chimeric peptide of a hormone-immunomimic peptide epitope fused in sequence with an immunogenic epitope. The hormone immunogenic peptide can be fused either directly to or through a spacer sequence to an immunogenic peptide epitope.

These fusion peptides combine at least one epitope of a target substance which may be non immunogen in its natural state with at least one immunogenic peptide sequence of suitable immunogenic proteins. The sequences of both target epitope and immunogen may be selected from the amino-terminal or carboxy-terminal region or both. A peptide also can be synthesized from the internal region of the peptide or protein. The fusion product may be acetylated at the amino-terminal end and amidated at the carboxy-terminal end of the peptide sequence.

An embodiment of the invention provides an anti-GnRH immunogen chimeric peptide construct comprising a suitable immunogenic epitope, such as, *e.g.*, short peptide sequences selected from the measles virus protein F (MVF), tetanus toxoid (TT), or malaria plasmodium falciparum CSP protein. The invention also provides for methods of immunization with a composition comprising a chimeric peptide with one or more GnRH epitopes.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the mean Anti-GnRH antibody titers obtained from rabbits using chimeric anti-GnRH Immunogens A through J, and as controls, Immunogens K and L as well as conjugate immunogen C GnRH:DT; and

Figure 2 illustrates the relationship between gross muscle reaction score and mean anti-GnRH Antibody Titer on GnRH Chimeras and Controls.

## DETAILED DESCRIPTION OF THE INVENTION

Chimeric peptides comprising GnRH mimicking epitopes have been constructed and useful in generating improved antibody titers.

Since self-antigen epitopes of gonadotropin releasing hormone (GnRH) are not inherently immunogenic the immune response may be aided by immunogenic constructs according to the invention wherein a target peptide epitope is located on the same synthesized peptide as is an immunogenic peptide epitope.

Several different chimeric peptides are described in Example 1.

EXAMPLE I

The peptide sequences combine a select promiscuous T-helper-epitope through an inserted short spacer peptide (e.g., 4-8 amino acids) with at least one target hormone peptide. Suitable spacers of this invention include but are not limited to the peptides comprising the following amino acid sequence, GPSL (see SEQ ID NO: 5); SSGPSL (SEQ ID NO: 6); and SSGPSLKL (SEQ ID NO: 7), which are inserted in the peptide chimera to isolate the three dimensional folding of the immunogenic peptide from that of the hormone peptide.

Promiscuous Th-epitope moieties from measles virus protein F (MSF) (sequence 288-302 aa, SEQ ID NO: 8), tetanus toxoid (TT) (sequence 947-967 aa, SEQ ID NO: 9, or sequence 830-844 aa, SEQ ID NO: 10) and malaria Plasmodium falciparum CSP protein (sequence 378-398 aa, SEQ ID NO: 11) are used in these constructs. The hormone immunomimic epitopes were attached to the N-terminal or the C-terminus of the spacer as shown below. All mammalian GnRH peptides including the human hormone, have the same sequence. The GnRH hormone immunomimic epitope sequence comprises 1-10 amino acids of mammalian GnRH when attached

to the aminoterminal peptide end and comprises 2-10 amino acids of mammalian GnRH when attached to the carboxyterminal peptide end. In addition, an immunomimic peptide comprising 13-16 amino acids of the mammalian GnRH comprise GnRH immunomimics peptides attached to both ends of the spacer, in order to increase the number of available GnRH antigenic epitopes. The different peptide chimera fusion immunogens in terms of antibodies produced are described below.

**Peptide 1.**

→ Measles virus protein F sequence (288-302aa) →

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NH2-K | L | L | S | E | I | K | G | V | I | V | H | R | L | E |

| | | | ← spacer → | | | | → GnRH (2-10 aa) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| G | V | E | G | P | S | L | H | W | S | Y | G | L | R | P |

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G-CONH2 (SEQ ID NO: 9 in the Sequence Listing)

**Peptide 2.**

→ Tetanus toxoid sequence (947-967 aa) →

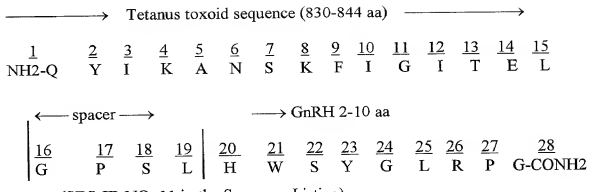
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NH2-F | N | N | F | T | V | S | F | W | L | R | V | P | K | V |

| → | | | | | | ← spacer → | | | | GnRH (2-10aa) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| S | A | S | H | L | E | G | P | S | L | H | W | S | Y | G |

| 31 | 32 | 33 | 34 |
|---|---|---|---|
| L | R | P | G-CONH2 |

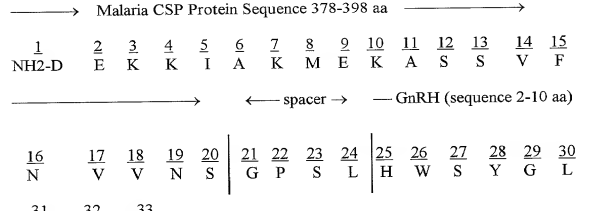
(SEQ ID NO: 10 in the Sequence Listing)

**Peptide 3.**




(SEQ ID NO: 11 in the Sequence Listing)

**Peptide 4.**




(SEQ ID NO: 12 in the Sequence Listing)

**Peptide 5.**

GnRH (sequence 1-10 aa) → ← spacer →

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p-E | H | W | S | Y | G | L | R | P | G | S | S | G | P | S |

← MVF-P (288-302 aa) →

| 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L | K | L | L | S | E | I | K | G | V | I | V | H | R | L |

| 31 | 32 | 33 | 34 |
|---|---|---|---|
| E | G | V | E-COOH |

(SEQ ID NO: 13 in the Sequence Listing)

**Peptide 6.**

→ GnRH (sequence 1-10 aa) → ← spacer →

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p-E | H | W | S | Y | G | L | R | P | G | S | S | G | P | S |

TT sequence 947-967 aa →

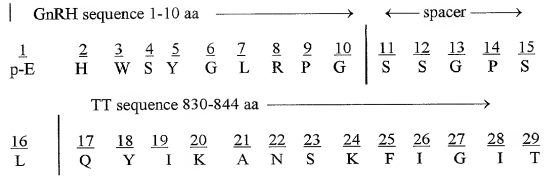
| 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L | F | N | N | F | T | V | S | F | W | L | R | V | P | K |

→

| 31 | 32 | 33 | 34 | 35 | 36 | 37 |
|---|---|---|---|---|---|---|
| V | S | A | S | H | L | E-COOH |

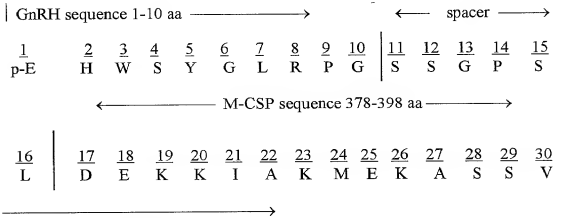
(SEQ ID NO: 14 in the Sequence Listing)

**Peptide 7.**




(SEQ ID NO: 15 in the Sequence Listing)

**Peptide 8.**




(SEQ ID NO: 16 in the Sequence Listing)

**Peptide 9.**

GnRH (sequence 1-10 aa) → | ← spacer →

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p-E | H | W | S | Y | G | L | R | P | G | S | S | G | P | S |

← MVF-P (288-302 aa) →

| 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L | K | L | L | S | E | I | K | G | V | I | V | H | R | L |

← spacer → | → GnRH (2-10 aa)

| 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E | G | V | E | G | P | S | L | H | W | S | Y | G | L | R | P |

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G-CONH2 (SEQ ID NO: 17 in the Sequence Listing)

**Peptide 10.**

→ GnRH (sequence 1-10 aa) → | ← spacer →

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p-E | H | W | S | Y | G | L | R | P | G | S | S | G | P | S |

TT sequence 947-967 aa →

| 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L | F | N | N | F | T | V | S | F | W | L | R | V | P | K |

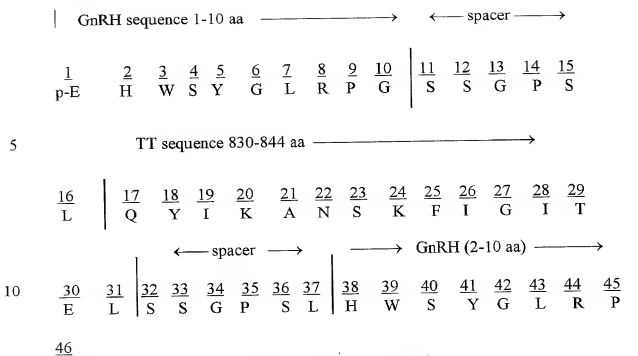
→ ← spacer → GnRH (2-10) →

| 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| V | S | A | S | H | L | E | G | P | S | L | H | W | S | Y | G |

| 47 | 48 | 49 | 50 |
|---|---|---|---|
| L | R | P | G-CONH2 |

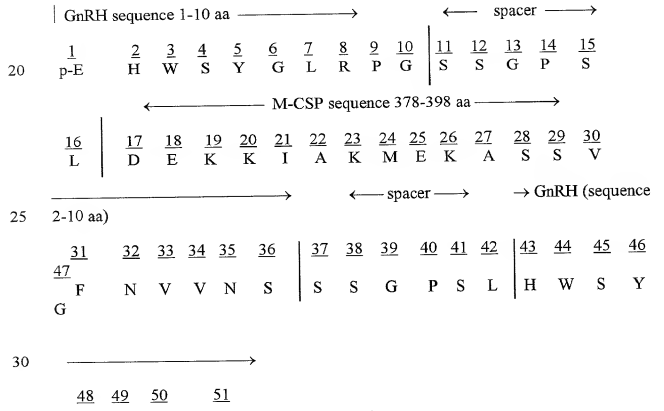
(SEQ ID NO: 18 in the Sequence Listing)

**Peptide 11.**




(SEQ ID NO: 19 in the Sequence Listing)

**Peptide 12.**



L R P G-CONH2 (SEQ ID NO: 20 in the Sequence Listing)

**EXAMPLE II**

Immunogenicity tests were performed with five chimeric peptide immunogens against GnRH. Each chimeric peptide contained one region encoding an epitope to be recognized by helper T-cell and a second region encoding an immunomimic of GnRH, to serve as the target for the antibody response. The chimeric peptide immunogens were formulated to deliver 100, 250 or 500 μg doses of peptide with 3μg norMDP, in a water in oil emulsion. Control immunogens were prepared to deliver 500 μg of mammalian GnRH (1-10) Ser1 peptide (which is normally linked to an immunogenic carrier to impart immunogenicity), with and without norMDP (3 μg), in the same emulsions. The immunogens were given intramuscularly to rabbits in three injections, on days 0, 14 and 42. An ELISA procedure was used to measure the resultant anti-GnRH antibody responses in sera collected at 14-day intervals over the course of the immunization. Injection site reactions were assessed by visual and microscopic evaluations on day 84.

The following materials were used in the immunogenicity tests. The five immunogens of GnRH chimera peptides tested were selected from the aforementioned Peptide 1 through 16.

1. GnRH chimera 1 {MVF (Measles Virus Protein F)} "Peptide 1" (MW 3427.17)
2. GnRH chimera 2 {TT-3 (Tetanus Toxoid Epitope 3)} "Peptide 2" (MW 3886.52)
3. GnRH chimera 3 {TT-2 (Tetanus Toxoid Epitope 2)} "Peptide 3" (MW 3132.6)
4. GnRH chimera 4 {MCSP (Malaria Circumsporozoite Protein)} "Peptide 4" (MW 3632.2)
5. GnRH chimera 6 (TT-3, N-ter GnRH) "Peptide 6" (MW 4172.7)
6. D17 Peptide ("GnRH (1-10) Ser 1")

For testing the GnRH chimeric peptide immunogens were formulated at concentrations listed below in Table 1. Each injection volume was 0.2 ml/dose (see Table 2).

Table 1. GnRH Chimera and Control Immunogen Formulations

| Immunogen | Chimeric Peptide | Concentration of Peptide in Emulsion (mg/ml) | Peptide Dose (µg/dose) | Concentration of norMDP in Emulsion (mg/ml) | norMDP Dose (µg/dose) |
|---|---|---|---|---|---|
| A | Peptide 1 | 2.5 | 500 | 0.015 | 3 |
| B | Peptide 2 | 2.5 | 500 | 0.015 | 3 |
| C | Peptide 2 | 1.25 | 250 | 0.015 | 3 |
| D | Peptide 2 | 0.5 | 100 | 0.015 | 3 |
| E | Peptide 3 | 1.25 | 500 | $7.2 \times 10^{-3}$ | 3 |
| F | Peptide 4 | 2.5 | 500 | 0.015 | 3 |
| G | Peptide 6 | 2.5 | 500 | 0.015 | 3 |
| H | Peptide 2 | 2.5 | 500 | 0.015 | 3 |
| I | Peptide 3 | 1.25 | 500 | $7.2 \times 10^{-3}$ | 3 |
| J | Peptide 2 & 3 | 0.625, each peptide | 250, each peptide | $7.2 \times 10^{-3}$ | 3 |
| K | D17 Peptide | 2.5 | 500 | 0.015 | 3 |
| L | D17 peptide | 2.5 | 500 | - | - |

The GnRH chimeric immunogenic compositions and control immunogens were formulated under clean conditions in the combinations shown in Table 1. The test materials were sterile bottled and stored under refrigeration (2-8°C).

New Zealand White female rabbits were immunized with GnRH chimera and control immunogens as shown in Table 2. Injections were given to each rabbit on days 0, 14 and 42 in dose volumes of either 0.2 ml or 0.4 ml. All immunogens were given IM, at injection sites tattooed for later identification.

To assess immunogenicity, sera were obtained from each rabbit every 14 days until day 84. Anti-GnRH antibody titers were measured in the sera samples by a direct binding ELISA. All values, with the exception of those for immunogen 6, are expressed relative to a reference standard rabbit anti-GnRH serum reference titer of 5,000. Titers of sera against Immunogen 6 (Peptide 6 N-terminal specific antibodies) were expressed relative to the reference standard rabbit anti-GnRH serum Ser 10(11) reference titer of 20,000.

Although the original study had two rabbit groups, the protocol was later amended to add two more groups (n=4), 3 and 4, with amounts of 250 μg and 100 μg of GnRH chimera 2 (TT-3) (Peptide 2), each with 3 μg of norMDP.

Table 2. Example II: Immunization Schedule

| Rabbit Group Number | N* | Peptide(s) | Injection Volume (ml/dose) |
|---|---|---|---|
| 1 | 4 | Peptide 1 500 μg | 0.2 |
| 2 | 4 | Peptide 2 500 μg | 0.2 |
| 3 | 4 | Peptide 2 250 μg | 0.2 |
| 4 | 4 | Peptide 2 100 μg | 0.2 |
| 5 | 4 | Peptide 3 500 μg | (2 x 0.2/site)** |
| 6 | 4 | Peptide 4 500 μg | 0.2 |
| 7 | 4 | Peptide 6 500 μg | 0.2 |
| 8 | 10 | Peptide 2 500 μg | 0.2 |
| 9 | 10 | Peptide 3 500 μg | (2 x 0.2/site)** |
| 10 | 6 | Peptides 2 & 3, 250 μg each | (2 x 0.2/site)** |
| 11 | 4 | D17 peptide (500 μg) with norMDP | 0.2 |
| 12 | 4 | D17 peptide 500 μg | 0.2 |

* N=number of rabbits per group

** Peptide 3 did not dissolve at higher concentrations, therefore injection volumes were doubled to deliver 500 μg/dose of total peptide.

Since GnRH chimera peptide 3 ("Peptide 3") (TT-2) was not found soluble at 9.412 mg/ml in aqueous phase, the original protocol was amended to reduce the concentration in half (4.706 mg/ml) and double the dose volume to maintain 0.2 ml

volume per injection (2 x 0.2 ml/site). Injection #3 was delivered on day 42.

Titers obtained for the individual serum samples are given in Table 3A/B/C, and mean titers for all groups are plotted in Figure 1, respectively. In the initial tests, all rabbits responded to the chimera peptides with the production of anti-GnRH antibody titers. Peptide 3 or GnRH chimera 3 (TT-2) induced significantly higher antibody titers in comparison with the other chimera peptides. Peptide 2 or Chimera 2 was most immunogenic at the 500 μg dose (Immunogen B), with the 100 μg (Immunogen D) and 250 μg (Immunogen C) doses inducing weaker titers. Chimeras 2 (Immunogen B) and 3 (Immunogen E) induced high antibody titers in the initial tests (n=4) relative to titers induced by GnRH:DT; however, these titers were lower in the repeat studies (n=10, Immunogen H where the response rate was quite variable, and Immunogen I, respectively).

A combination of Chimeras 2 and 3 (Immunogen J), at 250 μg dose of each (half the dose used in rabbits injected with the individual peptides) induced high titers of anti-GnRH antibody. Chimeras 1 (Immunogen A), 4 (Immunogen F) and 6 (Immunogen G) were not as potent as the GnRH:DT conjugate formulated in Montanide ISA 703 (as historical control included in Figures 1 and 2). It should be noted that Peptide 6 or GnRH chimera 6 (TT-3 in aminoterminal position) titers were measured using an N-terminus specific reference standard, therefore a statistical comparison of these titers with other chimera peptides was not performed. Nevertheless, Peptide 6 was concluded not to be an effective immunogen. Very low anti-GnRH antibody titers were induced by D17 peptide adjuvanted with norMDP (Immunogen K), while without norMDP (Immunogen L), the D17 peptide emulsion was not immunogenic.

Gross pathology of injection sites was assessed on all rabbits on day 84. The evaluation was scored on a scale of 0-3, where a score of 0 indicated normal tissue appearance and 3 indicated the presence of extensive tissue inflammation. Scores of 1 or 2 were judged intermediate levels of local reaction.

## Table 3A. Example II: Anti-GnRH Antibody Titers for GnRH Chimeras

| Immunogen | Injection→ / Rabbit # | Injection 1 (Day 0) Day 0 | Injection 2 (Day 14) Day 14 | Day 28 | Injection 3 (Day 42) Day 42 | Day 56 | Day 70 | Day 84 |
|---|---|---|---|---|---|---|---|---|
| A | 1 | 0 | 274 | 3,276 | 8,845 | 12,500 | 20,600 | 13,200 |
| | 2 | 0 | 0 | 636 | 2,193 | 4,667 | 13,400 | 8,249 |
| | 3 | 0 | 0 | 198 | 512 | 731 | 1,392 | 1,166 |
| | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **Mean** | **0** | **69** | **1,028** | **2,888** | **4,475** | **8,848** | **5,654** |
| | **Median** | **0** | **0** | **417** | **1,353** | **2,699** | **7,396** | **4,708** |
| | **S.D.** | **0** | **137** | **1,522** | **4,081** | **5,729** | **9,878** | **6,213** |
| B | 5 | 0 | 8,201 | 20,500 | 37,400 | 34,500 | 62,100 | 76,800 |
| | 6 | 0 | 12,400 | 46,400 | 81,200 | 134,000 | 93,100 | 108,000 |
| | 7 | 0 | 507 | 22,300 | 91,800 | 75,000 | 50,600 | 28,400 |
| | 8 | 0 | 589 | 2,085 | 16,100 | 24,800 | 31,800 | 32,700 |
| | **Mean** | **0** | **5,424** | **22,821** | **56,625** | **67,075** | **59,400** | **61,475** |
| | **Median** | **0** | **4,395** | **21,400** | **59,300** | **54,750** | **56,350** | **54,750** |
| | **S.D.** | **0** | **5,886** | **18,181** | **35,838** | **49,632** | **25,705** | **37,953** |
| C | 9 | 0 | 0 | 536 | 1,325 | 6,631 | 7,267 | 5,033 |
| | 10 | 0 | 0 | 1,240 | 3,551 | 19,700 | 19,600 | 7,886 |
| | 11 | 0 | 0 | 719 | 16,800 | 12,800 | 16,800 | 11,200 |
| | 12 | 0 | 0 | 454 | 2,671 | 5,017 | 5,844 | 3,692 |
| | **Mean** | **0** | **0** | **737** | **6,087** | **11,037** | **12,378** | **6,953** |
| | **Median** | **0** | **0** | **628** | **3,111** | **9,716** | **12,034** | **6,460** |
| | **S.D.** | **0** | **0** | **353** | **7,201** | **6,679** | **6,844** | **3,328** |
| D | 13 | 0 | 2,952 | 8,320 | 869 | 87,200 | 47,300 | 39,700 |
| | 14 | 0 | 841 | 21,600 | 57,500 | 93,000 | 25,100 | 11,800 |
| | 15 | 0 | 141 | 1,759 | 4,373 | 7,732 | 6,670 | 5,198 |
| | 16 | 0 | 0 | 5,220 | 7,044 | 7,363 | 6,120 | 4,731 |
| | **Mean** | **0** | **984** | **9,225** | **17,447** | **48,824** | **21,298** | **15,357** |
| | **Median** | **0** | **491** | **6,770** | **5,709** | **47,466** | **15,885** | **8,499** |
| | **S.D.** | **0** | **1,363** | **8,674** | **26,822** | **47,721** | **19,450** | **16,546** |
| E | 17 | 0 | 1,382 | 15,500 | 140,000 | 79,900 | 136,000 | 105,000 |
| | 18 | 0 | 264 | 13,200 | 50,800 | 41,700 | 120,000 | 145,000 |
| | 19 | 0 | 471 | 13,000 | 98,900 | 95,700 | 111,000 | 131,000 |
| | 20 | 0 | 2,317 | 13,400 | 35,900 | 52,800 | 80,500 | 85,100 |
| | **Mean** | **0** | **1,109** | **13,775** | **81,400** | **67,525** | **111,875** | **116,525** |
| | **Median** | **0** | **927** | **13,300** | **74,850** | **66,350** | **115,500** | **118,000** |
| | **S.D.** | **0** | **941** | **1,162** | **47,423** | **24,703** | **23,332** | **26,713** |
| F | 21 | 0 | 296 | 3,189 | 2,638 | 2,165 | 2,751 | 3,365 |
| | 22 | 0 | 0 | 441 | 5,920 | 4,912 | 8,760 | 12,200 |
| | 23 | 0 | 0 | 484 | 6,350 | 6,333 | 7,900 | 7,512 |
| | 24 | 0 | 0 | 3,556 | 60,300 | 20,400 | 24,300 | 18,700 |
| | **Mean** | **0** | **74** | **1,918** | **18,802** | **8,453** | **10,928** | **10,444** |
| | **Median** | **0** | **0** | **1,837** | **6,135** | **5,623** | **8,330** | **9,856** |
| | **S.D.** | **0** | **148** | **1,687** | **27,716** | **8,151** | **9,301** | **6,582** |

**Table 3B. Example II: Anit-GnRH Antibody Titers for GnRH Chimeras continued)**

| Immunogen | Injection→ Rabbit # | Injection 1 (Day 0) Day 0 | Injection 2 (Day 14) Day 14 | Day 28 | Injection 3 (Day 42) Day 42 | Day 56 | Day 70 | Day 84 |
|---|---|---|---|---|---|---|---|---|
| G | 25 | 0 | 0 | 0 | 0 | 105 | 640 | 1,165 |
| | 26 | 0 | 0 | 0 | 0 | 0 | 131 | 141 |
| | 27 | 0 | 0 | 0 | 166 | 914 | 3,554 | 3,830 |
| | 28 | 0 | 0 | 0 | 191 | 387 | 1,265 | 1,510 |
| | **Mean** | **0** | **0** | **0** | **89** | **362** | **1,398** | **1,662** |
| | **Median** | **0** | **0** | **0** | **83** | **246** | **953** | **1,338** |
| | **S.D.** | **0** | **0** | **0** | **104** | **409** | **1,511** | **1,558** |
| H | 29 | 0 | 0 | 0 | 0 | 208 | 708 | 693 |
| | 30 | 0 | 0 | 1,257 | 1,475 | 2,800 | 2,374 | 2,313 |
| | 31 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 32 | 0 | 0 | 0 | 147 | 1,319 | 2,051 | 1,559 |
| | 33 | 0 | 204 | 3,713 | 8,696 | 11,900 | 14,100 | 11,200 |
| | 34 | 0 | 0 | 413 | 480 | ** | 16,900 | 14,700 |
| | 35 | 0 | 0 | 366 | 326 | 1,879 | 3,462 | 3,022 |
| | 36 | 0 | 0 | 0 | 0 | 200 | 410 | 555 |
| | 37 | 0 | 0 | 163 | 774 | 2,825 | 4,677 | 5,109 |
| | 38 | 0 | 2,787 | 8,027 | 7,742 | 41,700 | 63,200 | 62,900 |
| | **Mean** | **0** | **299** | **1,394** | **1,964** | **6,981** | **10,788** | **10,205** |
| | **Median** | **0** | **0** | **265** | **403** | **1,879** | **2,918** | **2,668** |
| | **S.D.** | **0** | **877** | **2,597** | **3,335** | **13,523** | **19,319** | **19,149** |
| I | 39 | 0 | 0 | 228 | 877 | 7,841 | 12,200 | 9,998 |
| | 40 | 0 | 0 | 2,568 | 5,522 | 27,000 | 29,600 | 17,000 |
| | 41 | 0 | 895 | 7,474 | 31,400 | 29,500 | 46,300 | 34,500 |
| | 42 | 0 | 0 | 1,560 | 3,280 | 10,800 | 12,000 | 11,500 |
| | 43 | 0 | 222 | 3,510 | 16,600 | 20,600 | 31,300 | 26,500 |
| | 44 | 0 | 0 | 5,825 | 22,500 | 27,000 | 36,200 | 37,900 |
| | 45 | 0 | 1,249 | 24,300 | 39,300 | 65,000 | 67,700 | 69,100 |
| | 46 | 0 | 498 | 5,208 | 7,243 | 8,877 | 13,500 | 16,800 |
| | 47 | 0 | 0 | 2,091 | 5,509 | 10,100 | 19,200 | 18,300 |
| | 48 | 0 | 0 | 4,072 | 7,937 | 14,600 | 26,300 | 48,400 |
| | **Mean** | **0** | **286** | **5,684** | **14,017** | **22,132** | **29,430** | **29,000** |
| | **Median** | **0** | **0** | **3,791** | **7,590** | **17,600** | **27,950** | **22,400** |
| | **S.D.** | **0** | **452** | **6,886** | **13,061** | **17,164** | **17,535** | **18,782** |
| J | 49 | 0 | 219 | 4,179 | 33,900 | 81,500 | 85,300 | 113,000 |
| | 50 | 0 | 8,659 | 100,00 | 193,000 | 242,000 | 169,000 | 129,000 |
| | 51 | 0 | 305 | 0 | 89,500 | 91,300 | 97,800 | 69,000 |
| | 52 | 0 | 1,071 | 14,800 | 26,600 | 30,600 | 27,500 | 19,300 |
| | 53 | 0 | 554 | 11,000 | 64,000 | 32,500 | 31,400 | 31,100 |
| | 54 | 0 | 1,940 | 16,300 32,700 | 86,400 | 70,500 | 65,600 | 68,800 |
| | **Mean** | **0** | **2,125** | **29,830** | **82,333** | **91,400** | **79,433** | **71,700** |
| | **Median** | **0** | **813** | **15,550** | **75,200** | **76,000** | **75,450** | **68,900** |
| | **S.D.** | **0** | **3,263** | **35,647** | **60,172** | **77,950** | **52,134** | **43,356** |
| K | C1 | 0 | 746 | 1,515 | 2,201 | 1,918 | 2,074 | 1,913 |
| | C2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | C3 | 0 | 134 | 590 | 953 | 998 | 1,238 | 1,768 |
| | C4 | 0 | 323 | 2,279 | 1,345 | 1,225 | 1,640 | 987 |
| | **Mean** | **0** | **301** | **1,096** | **1,125** | **1,035** | **1,238** | **1,167** |
| | **Median** | **0** | **229** | **1,053** | **1,149** | **1,112** | **1,439** | **1,378** |
| | **S.D.** | **0** | **325** | **1,005** | **913** | **793** | **893** | **878** |
| L | C5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | C6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | C7 | 0 | 0 | 0 | 0 | 107 | 0 | 0 |
| | C8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **Mean** | **0** | **0** | **0** | **0** | **27** | **0** | **0** |
| | **Median** | **0** | **0** | **0** | **0** | **0** | **0** | **0** |
| | **S.D.** | **0** | **0** | **0** | **0** | **54** | **0** | **0** |

**Table 3C. Example II: Anit-GnRH Antibody Titers for GnRH Chimeras**

| Immunogen | Rabbit # | Injection→ Injectio n 1 (Day 0) Day 0 | Injectio n 2 (Day 14) Day 14 | Day 28 | Injection 3 (Day 42) Day 42 | Day 56 | Day 70 | Day 84 |
|---|---|---|---|---|---|---|---|---|
| Control | C9 | 0 | 475 | 7,210 | 11,400 | 8,812 | 8,762 | 8,338 |
| | C10 | 0 | 1,588 | 9,253 | 20,100 | 28,500 | 34,800 | 32,200 |
| GnRHDT Conjugate in Emulsion = 0.5 mg/ml | C11 | 0 | 0 | 4,593 | 17,700 | 25,100 | 35,400 | 19,800 |
| Conjugate Dose = 100 µg | C12 | 0 | 194 | 3,647 | 7,900 | 13,900 | 12,900 | 11,800 |
| Dose Volume = 0.2 ml | C13 | 0 | 169 | 1,565 | 2,559 | 4,752 | 7,204 | 7,115 |
| | C14 | 0 | 651 | 3,965 | 3,755 | 8,277 | 13,700 | 7,179 |
| | C15 | 0 | 123 | 2,785 | 2,627 | 4,198 | 5,218 | 3,891 |
| | C16 | 0 | 353 | 4,910 | 13,800 | 26,700 | 43,600 | 30,600 |
| | C17 | 0 | 333 | 8,573 | 25,100 | 30,300 | 57,400 | 26,200 |
| | C18 | 0 | 188 | 2,171 | 2,622 | 7,314 | 8,207 | 8,404 |
| | **Mean, Group 5** | **0** | **407** | **4,867** | **10,756** | **15,785** | **22,719** | **15,553** |
| | **Median, Group 5** | **0** | **264** | **4,279** | **9,650** | **11,356** | **13,300** | **10,102** |
| | **S.D.** | **0** | **455** | **2,653** | **8,216** | **10,617** | **18,486** | **10,695** |

* test titers are read at 20,000 titer of the reference standard, lot 122298SHG2

The score data are summarized in Table 4, indicating that most of the visual injection site scores ranged from 0 to 1, indicating that the immunogens were generally well tolerated. Histologic readings of the injection site biopsies which were performed as of day 84 were in accord with the gross evaluation.

These experiments demonstrated that chimera peptides carrying a T-lymphocyte epitope and expressing an immunomimic of GnRH can be used to induce potent anti-GnRH antibody responses. Peptides bearing TT-2 and TT-3 T-lymphocyte epitopes, derived from TT, were more effective than the T-lymphocyte epitopes derived from MVF and MCSP. A combination of the TT-2 and TT-3 bearing chimeras was particularly effective. It was surprisingly found that the GnRH epitope had to be on the carboxyterminus of the chimeras to be immunogenic. Most injection site reactions were of an acceptable level. Overall, the response compared favorably with those induced by the GnRH:DT (previously named, D17-DT) conjugate, indicating that the synthetic peptides could potentially enhance the choice of effective immunogens and perhaps even replace the conjugate method for producing an active component of the GnRH immunogen.

## Table 4. Example II: Reaction Scores

| Immunogen | MEAN REACTION SCORES | | | | | | REACTION SCORES >1 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Injection 1 | | Injection 2 | | Injection 3 | | Injection 1 | | Injection 2 | | Injection 3 | |
| | SITE 1 | SITE 2 | SITE 1 | SITE 2 | SITE 1 | SITE 2 | SITE 1 | SITE 2 | SITE 1 | SITE 2 | SITE 1 | SITE 2 |
| A | 0 | | 0.5 | | 0.5 | | 0 | | 0 | | 0 | |
| B | 0.4 | | 1.1 | | 1.1 | | 0 | | 1 | | 1 | |
| C | 0.1 | | 0.5 | | 0.5 | | 0 | | 0 | | 0 | |
| D | 0.3 | | 0.4 | | 1.0 | | 0 | | 0 | | 1 | |
| E | 0.6 | 0.3 | 0.9 | 0.6 | 0.8 | 1.3 | 0 | 0 | 1 | 0 | 0 | 1 |
| F | 0.5 | | 1.1 | | 1.1 | | 0 | | 1 | | 1 | |
| G | 0.1 | | 0.3 | | 0.8 | | 0 | | 0 | | 0 | |
| H | 0.1 | | 0.3 | | 0.4 | | 0 | | 0 | | 0 | |
| I | 0 | 0.4 | 0.1 | 0.5 | 0.6 | 0.7 | 0 | 0 | 0 | 0 | 1 | 1 |
| J | 0 | 0.4 | 0.5 | 0.5 | 1.0 | 1.3 | 0 | 0 | 0 | 0 | 1 | 2 |
| K | 0.4 | | 0.4 | | 1.0 | | 0 | | 0 | | 1 | |
| L | 0.3 | | 0 | | 0.3 | | 0 | | 0 | | 0 | |
| Conjugate Ctl. | 0.4 | | 0.6 | | 0.9 | | 0 | | 0 | | 1 | |